PROJECT NUMBER: 1904
PROJECT TITLE: Tobacco Physiology and Biochemistry
PROJECT LEADER: H. Y. Nakatani
PERIOD COVERED: April, 1988

I. LOW NICOTINE STUDY

A. **Objective**: To investigate the biochemistry of the nicotine biosynthetic pathway at putrescine N-methyltransferase (PMT) and specifically to isolate PMT from tobacco root extracts.

B. **Status**: Extraction of the roots from the group ten plants were initiated to the 40-65 % ammonium sulfate stage; the pellets were resuspended in 200 ml of buffer plus 1.5 M NaCl (2).

The Sephacryl 200 (Pharmacia) column has been calibrated with molecular weight standards run in pairs and in one experiment with the complete complement of four standards (3,4). In the latter case, there was an anamolous elution volume for chymotrypsinogen which did not fall on the standard curve (4). The reason for this phenomenon is unknown. Various samples from the radial flow phenyl-Sepharose column have been applied to this column and the PMT activity was eluted with an estimated molecular weight of 56,000 +/- 6000 Da for 4 sets (3,4,5). A second Sephacryl column is being set-up in the laboratory with an LKB fraction collecting system (4,5). Both Sephacryl columns will be run using this system.

Recently the S-adenosyl-homocysteine affinity columns were not found to bind PMT, and a series of experiments were conducted with the various batches provided by Bethesda Research Lab. One type (lot no. 71101), having both free amino and carboxyl termini was found to bind PMT. The PMT was eluted with 1.5 M NaCl (1). Moreover, a reactive red (a triazine dye) affinity column was also found to bind PMT. The binding was rather weak, however, since most of the PMT was eluted by simple washing with dialysis buffer. The reactive red was assumed to bind to proteins requiring nicotinamide as cofactor (1).

Several attempts were made to elute PMT from DEAE-Sepharose with a pH gradient. The results were negative although 5 mM phosphate, pH 6.5 and 5mM citrate-phosphate, pH 4.0 were used to develop the gradient. In another experiment 5 mM phosphate, pH 6.5 was paired with 10 mM citric acid, pH 2.0. The actual environment associated with the binding of PMT to this support medium may not have been affected by the conditions applied.

Another PMT (HPMT2) active sample was delivered to Hazleton for boosting the antigen response of the inoculated mice. A Western blot examination of this sample with the mouse antibody from mouse #5 was conducted using the polyvinylidene difluoride (PVDF) membrane and the new miniblotting device (5). A positive response

was observed whereas no response was observed with the control serum (5).

A MagniSort M bead experiment using two levels of MagniSort beads was conducted with the HPMT2 sample. A decrease of approximately 40 % in the PMT-activity with the lower bead level was observed. In addition, PMT activity was "released" from the beads following 1 M $MgCl_2$ washing (5). These results certainly show promise for the antibody program.

The miniblotter was demonstrated at Hazleton for the examination of the third test-bleed against a Western blot with samples in 10 lanes (5). The examination could not have been conducted using their classical antibody detection methods since 5 ml of antibody would have been required.

C. **Plans**: The roots from the group 11, hydroponically-grown, tobacco plants will be harvested. The purification of PMT will continue with various chromatographic media. Antibody binding studies will continue using MagniSort-beads, Western blots and microtiter plates.

D. **References**:

1. Malik, V. S. Notebook No. 8542.

2. Dunn, R. L. Notebook No. 7899.

3. Sykes, A. Notebook No. 8526.

4. Yu, T. Notebook No. 8381.

5. Mooz, E. D. Notebook No. 8599.

II. ALTERNATE HUMECTANTS (PG/G-FREE SHEETS/CIGARETTES) (1)

A. **Objective**: To find a plasticizer/humectant system that provides acceptable sheet materials without glycols (PG/G).

B. **Results**: PG/G-free RCB was made at the BL Plant in April (2). Samples of the PG/G-free RCB sheet and samples of the PG/G-free RL (RLTC and RL150B) sheets that were made at Park 500 in March were submitted to Microbiology and the Analytical Research Division for testing.

C. **Plans**: 100% cigarettes will be made from the PG/G-free RCB and RL sheets, along with the controls. Second quarter plans for the Alternate Humectant Program for 1988 will be issued by the end of April.

D. **References**:

1. Mooz, E. D. Notebook No. 8599.

2. Mooz, Elizabeth D. Request for the BL Plant to Produce PG/G-free RCB in Support of the Alternate Humectant High Priority Program. Memo to Mr. W. G. Overstreet; 1988 April 14.

III. ALTERNATE HUMECTANTS (GLYCERINE-FREE SHEET/CIGARETTES) (1)

A. **Objective**: To provide acceptable glycerine-free (G-free) RLTC, RL150B, and RCB sheets and G-free cigarettes for the European market.

B. **Results**: Domestic, G-free Marlboro-type cigarettes were made from Park 500 and BL Plant G-free sheets, G-free ET and ESB, and G-free casings and flavors. The cigarettes, including a control, were given to Cigarette Testing and to Flavor Development for evaluation. A three-month survivability test was run in the Semi-works on G-free RL (RLTC and RL150B) sheet that was made at Park 500 in 9/87.

C. **Plans**: A POL test is scheduled to be sent out the end of April to compare a domestic, G-free Marlboro vs a domestic, control Marlboro.

D. **References:**

1. Mooz, E. D. Notebook No. 8599.